Report of Dr. Avery (assisted by Drs. Abernethy, Babers, Chow, Dubos, Goebel, Goodner, Horsfall, C. M. MacLeod, and Stillman)

Studies on the transformation of specific types of pneumococcus. (Avery and C. M. MacLeod.) Rarely in the case of a pathogenic microörganism is it possible to correlate many of its biologically specific and pathogenic characters with the activity of a single enzymatic function of the cell itself. This correlation, however, is particularly striking in the case of Pneumococcus. In this instance the bacterial cell possesses a potential system of synthesizing enzymes, the functional activity of which results in the elaboration of a specific substance which is structurally disposed about the periphery of the cell in the form of a capsule. The presence of the capsule confers a new and highly differential type-specificity upon the microörganism, and conditions its capacity to grow and multiply in the tissues of the infected host. This synthesizing function is most highly developed, and the product of its activity most pronounced in cells best adapted to growth in the animal body. On the other hand, cells in which this system of synthesizing enzymes has been spontaneously or experimentally inactivated by exposure to an unfavorable environment are not only deprived of their capsules and type-specificity, but also lose their invasive properties and become relatively non-virulent for animals highly susceptible to infection with the original encapsulated strain.

Important and essential as is the activity of capsular function to the parasitic career of the microörganism

in the living host, it is, however, not vital to the life of the cell growing outside the animal body. For capsule-free cells are still capable in artificial media of carrying on the vegetative processes of metabolism and multiplication after the capsule synthesizing function has been inhibited or suppressed. This retrogressive change from more complex to simpler cell structure, with the resultant loss of type-specificity and virulence, is intimately associated with, if not wholly dependent upon, the inactivation of the system of enzymes concerned in the synthesis of the cell capsule. The function of capsular synthesis may therefore be regarded as an adaptive mechanism or defense reaction whereby the cell seeks to protect itself against the resisting forces of the invaded host.

Thus, pneumococci may exist as encapsulated or non-encapsulated cells which, in terms of bacterial dissociation, are referred to respectively as the S and R forms. The cells of these two forms differ from each other in morphological structure, colony topography and biological behavior, and these differences are in each instance referable to a gain or loss in activity of the capsule-producing enzymes of the cell.

Under suitable cultural conditions, the change from one form of cell to the other is reversible ($S \rightleftarrows R$). A type-specific strain of pneumococcus, after serial transfers in a medium containing immune serum of the homologous type, is converted into the R form in which capsular development is suppressed. Similarly, the non-encapsulated, degraded R cells may, by cultivation in anti-R serum, be caused to regain all the distinguishing and type-specific properties which

characterized their parasitic antecedents. Under these condi-
tions, the reversion is invariably back to the homologous type,
and is associated with the acquisition of virulence and the
re-synthesis of a capsular polysaccharide identical in chemical
constitution and type-specificity with that elaborated by the
parent strain. Once this change in functional activity has
occurred, irrespective of whether the variation induced is
progressive or retrogressive in nature, the cells thus altered
breed true on subsequent cultivation until they are again
subjected to environmental conditions which cause reversal of
function and form. From this point of view, the processes of
dissociation may be regarded as a response of the cells to
environmental influences which favor either the activation or
inhibition of the capsule-producing enzymes.

More significant, perhaps, than this direct form of
reversible dissociation (S⇄R) is the phenomenon of transform-
ation of specific types of Pneumococcus. Griffith first showed,
by a special technique in mice, that encapsulated cells of one
specific type may be transformed into other specific types
through the intermediate stage of the R forms. Dawson sub-
sequently found that the transformation of specific types can
be brought about in vitro without the use of animal inoculation.
Later, Alloway succeeded in causing transformation by growing
R cells in a medium containing small amounts of a filtered
extract of S cells of a heterologous type. Under these condi-
tions the R cells, irrespective of type derivation, acquired
all the type-specific characteristics of S cells from which the
extract was prepared. Thus, it has been experimentally

demonstrated that it is possible not only to reactivate the
capsule-synthesizing enzymes of an R cell, but actually to so
orient and direct the synthesis that R cells, under the
influence of a specific stimulus, are caused to elaborate a
capsular polysaccharide having the same chemical constitution
and serological specificity as that of the type of S cells from
which the activating stimulus was derived.

The work begun by Dr. Alloway and continued last year by
Dr. Rogers is being actively carried on at present in collabora-
tion with Dr. MacLeod in an attempt to ascertain the nature and
properties of the transforming principle present in active
extracts of S cells. Extracts thus derived can be freed of
intact cells and cellular detritus by filtration through a
Berkefeld candle, without loss of activity. The active
principle passes the filter, however, only when the extract is
adjusted to an alkaline reaction (pH 8^2) before filtration.
From crude extracts a large amount of irrelevant protein and
other inert material can be removed by adsorption with charcoal
without appreciable diminution in the potency of the absorbed
extract. The active principle in these partially purified and
water-clear extracts does not pass through parchment or cello-
phane membranes on prolonged dialysis against running water.

The transforming principle in active extracts with-
stands heating at 60°-80° C. for 2 hours, but is completely
destroyed on shorter exposure to higher temperatures. The
potency of an extract is lost when the reaction of the solution
is adjusted to an acidity equivalent to or greater than pH 4.
On the other hand, the transforming principle is stable in

extracts at pH 9[4], and at this alkaline reaction resists heat-
ing to 60° C. for 45 minutes.

Observations of considerable significance have
recently been made concerning the effect of certain enzymes on
the transforming activity of potent extracts. Then S cells
are allowed to undergo spontaneous autolysis, the resulting
autolysate is no longer capable of causing transformation,
indicating that the active transforming principle is destroyed
by the autolytic ferments of the cell. All active extracts
contain varying amounts of the specific capsular polysaccharide
in solution. It was early discovered, however, that the
chemically purified specific polysaccharides are by themselves
devoid of transforming activity. Moreover, Rogers showed, and
MacLeod has since confirmed the fact, that active extracts of
Type III pneumococci, which have been treated with the specific
enzyme capable of decomposing the Type III polysaccharide, still
possess transforming activity although the presence of the
specific carbohydrate is no longer detectable by the precipitin
test.

Similarly, all active extracts contain varying amounts
of bacterial protein. However, extracts which have been digested
by crystalline trypsin and subsequently dialysed to remove the
hydrolysed products of digestion are as fully, if not more,
active than the original extracts before digestion and dialysis.
Unless it is subsequently shown that the split products of
enzyme action account for the activity of digested extracts, it
may be tentatively concluded that the transforming potency of
extracts is due to the presence of some constituent not

destroyed by trypsin or by the specific carbohydrase.

Whatever may ultimately prove to be the nature of the
transforming principle, one of its most striking characteristics
is the type-specificity of its action on R cells. The mechanism
involved in the phenomenon of transformation and the nature of
the activating principle are still undetermined. However, the
results indicate that all R cells, in which retrogression has
not advanced too far, possess a potential but inactive system of
enzymes capable of synthesizing any of the type-specific poly-
saccharides, the particular one produced being determined by
the specificity of the activating stimulus. Once the capsular
function has been specifically activated, the newly transformed
cells continue to synthesize the same capsular material and
retain the same type-specificity through innumerable transfers
on artificial media without the further addition of the
activating substance initially employed to induce the trans-
formation. From these same cells, after prolonged cultivation,
a transforming principle of the same specific nature and
activity can again be extracted in an amount greatly in excess
of that originally added to initiate the change.

The study is being continued with the hope that
knowledge of this important cellular mechanism may lead to a
better understanding of the principles involved with specific
transformation and induced variations of living cells, not
only of Pneumococcus, but also those of other biological
systems. Furthermore, the thought suggests itself that were we
in possession of knowledge pertaining to the nature of the
substances which serve as activators and inhibitors of the

capsule-producing enzymes, the knowledge gained might afford a

new approach to a specific attack directed toward the suppression

of the capsular function upon the activity of which the patho-

enicity of Pneumococcus depends.

The protective action of antipneumococcus serum in

mice (Goodner and Horsfall). Type-specific antipneumococcus

horse and rabbit sera have the capacity to protect mice against

fatal infection with pneumococci of the homologous type, even

when enormous numbers of the latter are administered with the

immune serum. There are, however, definite limitations in both

the amount of serum and the amount of culture which can be used

in order to demonstrate protection. An example of these

limitations is shown in Text Fig. 1, which includes results

with both rabbit and horse immune sera of approximately the same

agglutinative titers. In both instances there is a limiting or

titer zone in which the amount of immune serum appears to be

insufficient. In both instances, also, one observes the so-

called Schwellenwert or upper limit of culture beyond which it

appears to be impossible to protect mice irrespective of the

amount of serum. The most striking difference between the

results with the two sera is that whereas the immune rabbit

serum protected mice even when given in large amounts, the

administration of immune horse serum in similar quantities

resulted in a negative phase or prozone in which no protection

was obtained.

The Schwellenwert. The clue to the nature of the

Schwellenwert was found in a series of experiments which may

be summarized as follows:

Amount of serum (cc.)	Source of immune serum							
	Rabbit				Horse			
	Amount of culture (cc.)							
	0.4	0.2	0.1	0.05	0.4	0.2	0.1	0.05
0.4	●●●	●○○	○○○	○○○		●●●	●●●	●●●
0.2	●●●	○○○	○○○	○○○	●●●	●●●	●●○	●●○
0.1	●●●	●●○	○○○	○○○	●●●	●●○	●○○	○○○
0.05	●●●	●○○	○○○	○○○	●●●	●○○	○○○	○○○
0.025	●●●	●●●	●○○	○○○	●●●	●○○	○○○	○○○
0.0125	●●●	●○○	○○○	○○○	●●●	●●●	●●○	●○○
0.00625		●●●	●●○	○○○		●●○	●●○	●○○
0.003125			●●●	○○○		●●○	●●●	●●●
0.00156						●●●	●●●	●●●

●—Death
○—Survival

Fig. 1. Protection Tests with Type I Antipneumococcus Horse and Rabbit Sera.

Serum and culture administered simultaneously. Type I Pneumococcus culture possessed a virulence such that 0.000,000,01 cc. produced fatal infection in controls.

1. If, instead of administering serum and culture simultaneously, the former is given 18 hours in advance of the infecting organisms, a very large proportion of the antibody is obviously no longer available in the peritoneum. In spite of this loss, a considerable degree of protection is obtained if large amounts of serum are used, and it is especially noteworthy that under these conditions a number of mice can be protected against relatively large amounts of culture. The results suggest that the cellular make-up of the peritoneal fluid might have been altered by the presence of the foreign protein.

2. If intraperitoneal injections of sodium nucleinate are given 18 hours prior to the simultaneous administration of serum and culture, the protective action of antipneumococcus serum is markedly enhanced. The Schwellenwert undergoes a considerable shift in that the same amount of serum is now effective against larger amounts of culture.

Since it is known that the previous administration of sodium nucleinate brings about a considerable cellular response, it was considered possible that for each individual animal there is a true Schwellenwert related to the number and nature of the cells in the peritoneum at the time of injection of serum and culture.

After a thorough exploration of the possible technical approaches to the solution of this question, the following technique was adopted: Fifteen minutes after the injection of serum and culture, a sample of peritoneal contents was taken with a small capillary pipette and a smear made. From the

Gram stain the number of pneumococci and of cells, in many contiguous fields, were counted and the ratio of pneumococci to white cells determined. This ratio is obviously a function of the total number of white cells in the peritoneum, providing that the same numbers of pneumococci are injected in each instance. By means of this technique, an extensive study has been carried out, the results of which may be summarized as follows:

1. In mice of the same weight receiving the same amounts of immune serum, there appears to be a limiting ratio of pneumococci per white cell, and if this ratio is exceeded death is the inevitable result. In other words, mice with large numbers of white cells in the peritoneum are much more able to utilize the benefits of the immune serum than are those with few cells. These ratios serve as a basis for prediction of survival or death with an accuracy of about 85 per cent.

2. The limiting ratio in mice of the same weight is modified by the amount of immune serum. For example, with one particular lot of antipneumococcus horse serum, the best protection against 0.1 cc. of culture is obtained with 0.05 cc. Here the limiting ratio is approximately 7.0. With 0.0125 cc. of serum the limiting ratio is 5.4. With 0.2 cc. of serum the ratio is 4.0.

3. In a series of mice receiving the same amounts of serum, but differing in weight, the limiting ratio is a function of weight and becomes smaller as the weight increases.

4. With Dr. D. Miller, it has been possible to show that the latter finding is explained by the fact that very

small animals have a very high proportion of monocytes in the peritoneal fluid and vice versa. There are almost no polymorphonuclear leukocytes in the normal mouse peritoneum.

5. As an outgrowth of the foregoing, and in the light of certain other experimental facts, it can be inferred that both weight and peritoneal cell content can be in theory resolved into a single host factor, that is, the absolute number of monocytes in the peritoneum at the time of infective inoculation. These cells appear to constitute the first line of defense

To summarize, the Schwellenwert, or limitation of the number of pneumococci which the passively immunized animal can dispose of, is a numerical function of the relation of the number of microörganisms to the number of monocytes in the peritoneum at the time of injection.

The prozone. Referring to Text Fig. 1, it is of some interest that whereas 0.05 cc. of antipneumococcus horse serum protects against 0.1 cc. of culture, larger amounts are less effective. With 0.4 cc. no protection is obtained. This phenomenon is referred to as the prozone, and has been the subject of an intensive investigation. The results may be summarized as follows:

1. The extent of the prozone in the protection test is quantitatively correlated with the agglutinin titer, that is, immune sera with high agglutinin titers show a more extensive prozone than do those with low titers.

2. For each lot of antipneumococcus horse serum, there appears to be a definite amount which gives optimal protection in the range of larger amounts of culture. This

optimal amount is related inversely to the agglutinin titer.
Thus, one lot of serum with an agglutinin titer of 1-64 gives
optimal protection when 0.2 cc. is used; a second lot with
agglutinin titer of 1-256 with 0.05 cc.; while a third lot
(concentrated) with a titer 1-512 gives best protection in
0.025 cc. amounts.

3. Failure of protection in the prozone range is
associated with a marked decrease in the value of the
Schwellenwert. An example of this has already been cited.

4. Failure of protection in the prozone range is
associated with an apparent inhibition of phagocytosis. The
pneumococci, if unchecked, quickly multiply, hence the early
reaction on the part of the body is absolutely essential if
protection is to be achieved. In the following table are shown
results which bear on this subject:

	White cells showing phagocytosis during first hour Per cent	Pneumococci intra-cellular at end of first hour Per cent
No serum	6	8
Optimal protective amount of serum	20	62
"Prozone" amount of serum	6	2

It would appear that the excess of immune horse serum, by
inhibiting phagocytosis, permits the almost unchecked growth
of pneumococci.

5. Certain heterologous substances have been injected
with optimal amounts of immune horse serum. The addition of
normal rabbit serum or of egg white fails to alter the protection.

On the other hand, Type II antipneumococcus horse serum, concentrated antimeningococcus serum, lipemic rabbit serum and egg lecithin tend to block protection.

Since the only obvious common component of these unrelated inhibitory substances is lipoid, these findings suggested that it might be possible to remove the inhibitory element in immune horse serum by extracting the lipoids from it. This proved not to be the case, but this theory has led to an interesting series of findings which may be summarized as follows:

1. If raw antipneumococcus horse serum is extracted with alcohol-ether by a special technique which does not involve denaturation of the protein, the extracted serum fails to agglutinate pneumococci and does not give a precipitin reaction with the specific capsular polysaccharide. This result is not obtained with antipneumococcus rabbit serum similarly extracted.

2. The extracted horse serum still possesses an unaltered capacity to protect mice against fatal infection, even though the presence of antibodies cannot be demonstrated by agglutination or precipitation methods.

3. If extracted immune horse serum is injected intra-peritoneally into mice and the peritoneal fluid withdrawn after 30 minutes, the recovered fluid possesses the capacity to agglutinate pneumococci. This result suggests that the modified antibody combines with a substance in the peritoneal fluid. A similar "reactivation" can be brought about in vitro by the admixture of the extracted serum and lecithin under certain conditions.

4. These results suggest that the antibody of immune horse serum may consist of a loose combination of protein and lipoid, the protein carrying the specific combining groups. It is believed that the lipoid element may be responsible for the prozone. Extracted sera still give the prozone, but this may be accounted for by an in vivo recombination.

5. Antipneumococcus sera from various other animal species have been examined from this point of view. The results indicate that the rat and the guinea pig may be grouped with the rabbit, while the goat and the mouse are in the same class as the horse.

6. The results presented in this summary are a further demonstration of the wide differences in the properties of anti-pneumococcus sera derived from rabbits and horses. Previous observations have demonstrated radical differences in the morphological characteristics of the precipitates with the specific capsular polysaccharides (Schiemann and Casper), the ability to give the Quellung reaction with the pneumococcus capsule (Neufeld and Tulcynski), the property of fixing complement with the capsular polysaccharides (Zinsser and Parker), etc. It is believed that the present work may furnish the clue necessary to the explanation of these differences.

To summarize, it has been shown that high titered antipneumococcus horse serum in amounts above the optimum fails to protect mice, whereas this is not true with antipneumococcus rabbit serum of equivalent antibody content. The results indicate that this failure to protect is due to an inhibition of the phagocytic mechanism by some element of the immune serum

which has not been absolutely identified, but which is believed
to be lipoid in nature.

The relationship of uronic acid to the biological
specificity of bacterial polysaccharides (Goebel). In previous
reports we have described the results of studies in which
certain structural and configurational differences of the
carbohydrate radicals of artificially compounded antigens have
been correlated with changes in immunological specificity.
These studies have been directed particularly to explaining the
factors which determine the specificity of carbohydrates. It
has been found that known differences in the chemical constitu-
tion of the carbohydrate radicals of synthetic antigens are
sharply reflected in the serological specificity of the anti-
sera to which they give rise in the animal body. This work has
now been extended to the naturally occurring bacterial poly-
saccharides. The present report deals with an explanation of
the cross serological reactivity of the capsular polysaccharides
of Types III and VIII pneumococcus on the basis of certain
similarities in the constitutions of the two polysaccharide
molecules.

The specific polysaccharide of Type VIII pneumococcus
has been isolated as a nitrogen-free carbohydrate, and shown to
be built up of units of glucose and glucuronic acid in the ratio
of seven molecules of hexose to two of uronic acid. When the
polysaccharide is hydrolyzed with dilute mineral acid, the
hydrolysate is found to contain glucose and an aldobionic
acid---a glucose glucuronide.

It has previously been shown that the specific poly-saccharide of Pneumococcus Type III is likewise built up of molecules of glucose and glucuronic acid in the ratio of 1:1. When the polysaccharide is hydrolized with dilute acid, there is found in the hydrolysate an aldobionic acid, which has been likewise shown to be a glucose glucuronide.

It has been experimentally demonstrated that if the free hydroxyl groups of a bacterial polysaccharide are substi-tuted with a chemical radical, the resulting derivative retains its capacity to react in homologous antiserum. If, on the other hand, the highly polar carboxyl group is covered with an ester grouping, the corresponding derivative loses entirely its serological specificity. It is believed, therefore, that the uronic acid constituent, and particularly the spatial relation-ship of its dominantly polar carboxyl group to other groups in the carbohydrate molecule, plays a very important role in determining the type-specificity of a bacterial polysaccharide. If it were possible, therefore, to show that the aldobionic acids of the specific polysaccharides of Types III and VIII pneumococcus were identical, then the cross serological reactivity of these two substances would be established on a definite chemical basis.

The aldobionic acids derived from the specific poly-saccharides of Types III and VIII pneumococcus are both amorphous substances. It therefore becomes necessary to prepare a crystalline derivative of these two sugar acids in order to establish either their identity or dissimilarity. Consequently,

the methyl esters of each were prepared, which, on subsequent
acetylation yielded the corresponding crystalline heptacetates.
When obtained in a state of purity these two derivatives were
found to be identical in crystalline structure, melting point
and specific optical rotation. It may be concluded, therefore,
that the structural unit of the specific polysaccharide of .
Pneumococcus Type III is identical with one of the structural
units of the polysaccharide of Type VIII. It is believed,
therefore, that the cross serological reactivity of these
naturally occurring bacterial polysaccharides may be explained
on the basis of the identity in structure of the aldobionic
acid nucleus common to both carbohydrates.

It has been found that the specific enzyme which
hydrolyzes the capsular polysaccharide of Type III Pneumococcus
is elaborated not only when the organism from which the enzyme
is derived is grown in the presence of the polysaccharide, but
is likewise elaborated when the Type III aldobionic acid itself
is used as a substrate. Thus it would appear that if the
structure of the aldobionic acid were established it should be
possible to produce synthetically a specific substrate for the
production of the enzyme. The problem of synthesizing the
aldobionic acid involves, however, not only a knowledge of its
structure, but a knowledge of the chemistry of glucuronic acid
as well.

Because the appropriate source material---an
acetohalogen derivative of glucuronic acid---has never been
satisfactorily prepared, the synthesis of glucuronides has
heretofore never been achieved. In collaboration with

Mr. Babers we have attempted to prepare a series of derivatives of glucuronic acid to be utilized for the syntheses of glucuronides and aldobionic acids as well. Thus it has been found that when glucuron, the lactone of glucuronic acid, is acetylated, there are obtained two isomeric triacetates from which a crystalline chlorodiacetyl glucuron can be prepared in excellent yields. It has been found that when this halogen derivative is condensed with methyl alcohol in the presence of silver carbonate the corresponding crystalline methyl glucuronide is obtained. From a study of the kinetics of hydrolysis of this derivative in the presence of extremely dilute acid, and from the fact that alkaline hydrolysis removes but one of the acetyl groups, it must be concluded that this derivative does not represent a true glucuronide with the structure:

I
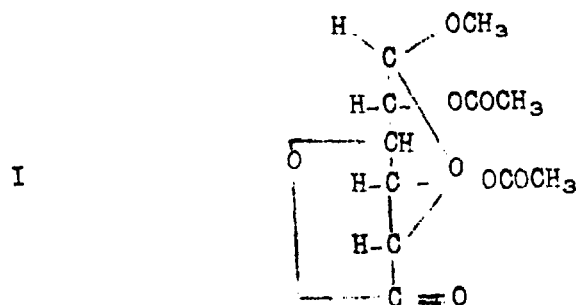
but a derivative of ortho acetic acid having the following structure:

II
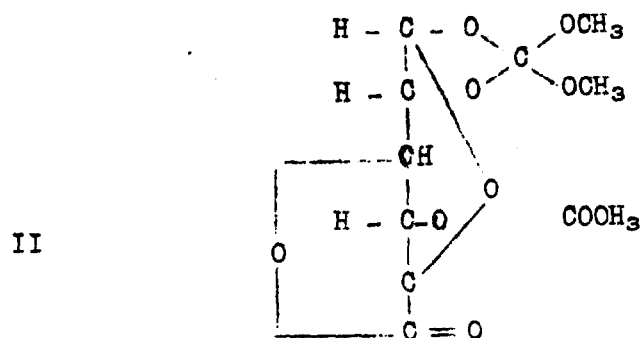
In view of the fact that the aceto halogen derivative
of glucuron failed to yield a true methyl glucuronide, it was
thought that glucuronic acid itself might furnish the appropriate
derivative. Consequently, glucuronic acid was converted into
the methyl ester, which, in turn, yielded the two isomeric
α and β tetracetates upon acetylation. The latter derivatives
were readily converted into the corresponding acetochloro
derivative, from which the triacetyl methyl ester methyl
glucuronide was prepared. Like the corresponding derivative
of glucuron, however, this methyl glucuronide was found to be
an orthoacetate, and not a true glycoside.

From the results of the above experiments, it would appear
that it is impossible to synthesize the true glucuronides either
from the halogen derivative of glucuron, or from that of the
methyl ester of glucuronic acid. Further experimental work,
however, has revealed the fact that if the molecular weight of
the aglucon is increased, and if diacetyl chloroglucuron is
condensed with the appropriate alcohol, such as nitrobenzyl
alcohol, a glucuronide is obtained having the structure of a
true glycoside. Furthermore, it has just recently been found
that if the β tetracetyl methyl ester of glucuronic acid is
warmed with titanium tetrachloride in chloroform solution, a
second crystalline triacetyl chloro glucuronic acid methyl
ester is secured which is isomeric with the chloro derivative
prepared as previously described. On the basis of certain
knowledge pertaining to the structure of this second halogen
derivative, it may be anticipated that the latter compound will
likewise yield the true glycosides of glucuronic acid. Thus,

the synthesis of the aldobionic acid of Pneumococcus Type III specific polysaccharide awaits the determination of the structure of this biose.

The synthesis of the acidobionic acid common to the specific polysaccharides of Types III and VIII Pneumococcus has not only a practical basis---that of supplying a synthetic substrate for enzyme production, but a theoretical interest as well. By synthesizing the fundamental building stone of the polysaccharide of Pneumococcus Type III, it is our hope eventually to prepare an antigen, truly artificial, and bearing to the Type III Pneumococcus no relationship whatsoever save one of chemical structure, which will give rise in the host to specific immunity against infection with Type III pneumococci.

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